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# IMPLEMENTING CRYOGENIC STORAGE OF CLONALLY PROPAGATED PLANTS

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## Summary

Methodology for plant germplasm cryopreservation was created in the 1970s, expanded in the 1980s, and implemented at the end of the 20th century. Translating experimental techniques into routine cryostorage of a clonal collection requires attention to details beyond those normally required for methods development. Early decisions include the choice of accessions to be stored, number of each accession per storage unit, number of replicates, location of storage, viability testing, record keeping and proper control groups. Emphasis should be placed on selecting a secure storage site and compiling complete records needed for the recovery of plant material. Secure remote storage, duplicate locations, and secure, accurate records are all important in ensuring the safety and usefulness of base collections. Evaluation of cryostored collections should be initiated to determine the longevity of plants and stability of storage conditions. Collections of several clonal genera are now stored in liquid nitrogen and more are in progress worldwide. These base-storage collections of clonal germplasm provide security for safeguarding long-term access to genetic diversity that is vital to food security and to continued improvement of many clonally-propagated agricultural crops.

**Keywords**: germplasm, cryopreservation

## INTRODUCTION

Cryopreservation is considered the best option for the long-term storage of clonal germplasm (6). Cryopreservation techniques developed over the last 25 years are now advanced to the stage where they can be implemented for useful storage of germplasm. Techniques of controlled freezing, vitrification, encapsulation-dehydration, dormant bud preservation, and combinations of these are now available for use with plant genotypes in hundreds of species. However, few labs are actually putting the techniques to large-scale use. Initial implementation of procedures can be daunting for facilities that are already low oil financial and human resources. The steps of experimental protocols tested on one or a few genotypes in a genus may not work for the range of germplasm available in national or even breeder collections. Initial steps must be taken to adapt these protocols and set up procedures for testing, screening, and ultimately storing the range of genotypes in each collection (26). Although testing to date has shown no variation due to cryopreservation protocols, additional testing over time is needed to determine the long-term stability of the system.

Present day technology and the large base of research 'into cryopreservation methods over the last 25 years have produced several protocols for the preservation of apical meristems (6).

1. **Controlled Freezing** (slow freezing, two-step freezing). Meristems are pretreated in cryoprotectants and frozen at < 1 °C/min to about -40° C, then plunged into liquid nitrogen (LN)(18, 24, 31)



2. **Vitrification** (rapid cooling). Several protocols and cryoprotectants are available. After a relatively brief cryoprotectant pretreatment the vials are directly plunged into liquid nitrogen (29, 37).

3. **Dehydration-Encapsulation.** Apices or stem tips are encapsulated in alginate and dehydrated osmotically and with airflow before direct immersion in liquid nitrogen (5).
4. **Combinations** of the above techniques are also available (30).
5. **Dormant bud preservation.** Dormant wood collected from trees during the coldest winter months are given additional chilling, dehydrated gradually by slow cooling, and held in LN vapor. Thawed buds are grafted or budded onto rootstocks for recovery (7).

## ISSUES INVOLVED IN IMPLEMENTING LIQUID NITROGEN STORAGE

*Initial decisions*

Now that many successful techniques are available and already tested on numerous genotypes, the time has come to start storing germplasm. Implementing storage requires many considerations. The present selection of cryopreserved plants vary from randomly chosen selections to carefully selected clones that represent morphological variation and unique geographic criteria sometimes referred to as "core" collections (9, 16, 26, 28). The curatorial decision regarding what to store generally starts with at-risk plants i.e. those that are likely to be lost to disease, insect pests, or environmental conditions at the field site. These decisions will vary with crop type, location of the field genebank, and economic importance of each crop. The form of plant to store is also an issue, and pollen, shoot apices, dormant buds, excised embryonic axes, or somatic embryos may be the form of choice depending oil the species involved (1). The next decisions involve logistics of storage. How many samples of each accession should be stored? The number of propagules will vary with the plant type and the recovery potential. Ideally enough propagules should be stored in each container to produce several living plants and enough containers to allow for several recoveries over time. If periodic testing is anticipated, then additional vials should be included for that purpose so that the vials in the base collection remain untouched (see long-term monitoring below).

*Developing a testing and storage protocol*

Once the initial decisions are made on accessions to be stored and how many of each, the testing protocol must be established. The amount of initial testing varies greatly from facility to facility. If the development of methods involved several species and cultivars, then less testing may be needed at the storage phase. There are two schools of thought on testing. The first system uses several storage trials and their controls as the testing phase. In this scenario a lab might Store five vials of 10 propagules and use one as the control. If the control recovers with a high percentage, then four vials remain in storage. If recovery of the control is low, then another group is stored to increase the number of viable propagules in storage (32). The second method stores a small number of propagules in two vials and thaws both after a short time. If tile regrowth percentage is >40%, then another group is processed and stored. If the percentage is lower, improvements are made to the culture or cryopreservation protocols to improve performance before storage (9, 26).

Both internal and external controls are needed for any storage protocol. Internal controls include regrowth of plants at critical stages of the protocol, For meristem cryopreservation this might occur after dissection from the mother plant, after cryoprotection, and after LN exposure. If cryopreserved vials are transferred from one storage container to another or from one location to another, a control vial should be regrown to monitor the effect of those transfers. Proper techniques for inserting and removing vials from storage containers should be developed before storage begins. Improper handling at the storage or removal point can easily kill propagules.

*Storage location*

For long-term storage the physical location of the storage dewar is very important. As a base collection of important germplasm, the dewar should be located at a site remote from the field genebank if at all possible. The storage site needs to be secure and under the control of dependable management to insure that LN is added to the dewar as needed. Alarms should be installed to monitor the LN level in the dewars and ensure constant temperature in the storage container. The use of a remote storage location is especially important when field collections are at risk due to environmental or political problems (earthquake, hurricane, flood, volcano, or civil unrest).

*Long-term monitoring*

Monitoring of viability of cryopreserved collections is problematic. Ideally storage would be in a dewar that is not disturbed, filled regularly, and accessed only rarely. In this ideal case viability should remain similar to control percentages and little testing is required. If storage is in a general purpose dewar that is often used, regular monitoring may be required. Since little data is available on the effect of dewar use on the life of stored propagules, it is difficult to propose an adequate testing scheme. The best advice might be to designate a dewar for storage and ensure that it is not used for other purposes. Dewars with long holding times (one to two years) are now available and would be ideal for base collections.

*Cryopreservation and storage records*

Germplasm systems require information on the origin of each plant (passport information), and cryopreserved propagules must be linked to that original plant accession. Important cryogenic information must be linked as well since the propagules are to be retrieved 50, 100, or 500 years in the future, Each accession must have information on preparation, pretreatment, cryopreservation method, thawing method, and the recovery medium. Especially critical to recovering the germplasm are thawing methods and recovery media. These two items should be readily accessible in the accession database for easy access by future curators wishing to recover plants. Complete protocol information could be in a secondary database as it is not critical to recovering plants, but may be of scientific interest.

## LONG-TERM STORAGE STABILITY

Plant tissues were first cryostored in the 1970's and initial tests with short term storage of pea (2 yr), strawberry (10 yr) and potato (I yr) meristems indicate no loss of viability (14, 19, 34). Kartha et al. noted fluctuations in strawberry meristem viability after various LN storage periods, but were attributed to differences in the physiological status of the meristems used in different experiments, not the result of LN storage. Three generations of field grown strawberries produced runners, exhibited vigorous growth, and produced normal fruit (17). Towill (34) recovered normal potato plants from cryopreserved meristems (34) and Reed et al. (25, 27) observed that strawberry plants of 10 cultivars stored in LN and evaluated in the field all produced normal leaves, flowers, and fruit.

Phenotypic changes arising in plants grown in tissue culture are termed somaclonal variants (21). These variations are most common in plants regenerated from single cells or callus. Variation occurs, but is not common, in micropropagated or meristem propagated plants derived from existing meristems. If care is taken in the clonal multiplication of plant materials, the amount of variation in propagules should be no different from field plants.

Genotypes that often produce mutations/sports will also produce them *in vitro* (banana is a good example of a variable genus). Germplasm scientists who are familiar with the plants to be cryopreserved are able to recognize genetic variability before or after the cryopreservation process. The establishment of descriptor characteristics for each genotype allows for rouging of off types upon regrowth. Any genotype with a known tendency to produce sports/mutations should be flagged in the database and propagules carefully selected for storage. Field and genetic analyses are needed to determine whether instability is a problem, however several studies have shown little evidence for concern (10- 13, 20, 35).

## PHYSICAL AND BIOCHEMICAL STABILITY

Another factor relating to viability of propagules stored for long periods involves the physical and biochemical stability of the system. Vitrified solutions are known to crack from physical shocks or in response to certain warming procedures. Thermal-stress induced fractures of biological materials may cause serious damage to stored samples. Slow cooling rates minimize thermal stress from non-uniform temperature distribution, and cryoprotectants reduce stress by changing the microstructure of the ice formed (8). Fractures can also occur as random events during cryopreservation (22). Fractures typically occur in large organs such as whole seeds and are less common in cell suspensions and meristems. Most reports of physical cracking are with animal organs (liver slices, veins, and arteries) rather than plant specimens.

The kinetic stability of red blood cells and liposomes was found to decrease at a predictable rate if stored at temperatures above the glass transition (33). The stability of vitrified biological materials held at temperatures above the glass transition was predicted to decrease by a factor of 10 for every 15'C. As a glassy (vitrified) system cools toward its glass transition temperature, cellular processes become increasingly slow or nearly arrested because of high viscosity. Molecular movement is also slowed. Additional studies of the relationship between the glass transition temperature and the stability of cryopreserved organisms are needed to adequately predict storage life of biological collections (33).

Cryopreserved samples held in dewars are exposed to warming and cooling cycles as other samples are added or removed. There are no studies that quantify the effects of these temperature variations on storage life. Possibly we could apply the stability principles discussed above to this phenomenon and hypothesize that these fluctuations in temperature would impact the storage life of a cryopreserved sample. It is also not known if stability varies among the different cryopreservation procedures.

Biochemical stability of cryopreserved plant cells is supported by the analysis of many cell cultures producing secondary products (2, 3, 38). Studies of the genetic stability of cell and meristem cultures also validate the stability of those systems (10-13).

## IS ONE PROTOCOL BETTER THAN THE OTHERS ?

Some of the relative advantages and disadvantages of cryopreservation protocols for long-term storage are evident (Table I ), however many may not be evident for many years. At present the choice of techniques is related to the needs of the facility involved. Personnel, equipment, expertise, plant type, and available facilities may influence which technique is most appropriate.

Germplasm storage

Curators will need to determine the amounts and types of germplasm to be stored based oil plant characteristics. A rough estimate of the number of propagules needed should be based on the percent recovery following cryopreservation, the ease of regrowth of the plant, and tile number of times samples will be removed from storage. For most clonally propagated crops recovery of five or more plants from a vial would provide adequate material for micropropagation.

If the cryopreserved control recovery were 50% or more, then 10 meristems per vial would be adequate for long-term storage, If more individuals are desired or the recovery percentage is lower, 25 meristems per vial might be warranted. Most accessions could be stored as four or five vials, thus allowing four or five uses over 100 years. Any accessions used for viability testing over time should include 10 or more vials.

Table 1. Advantages and disadvantages of some commonly used cryopreservation techniques.

| **Technique** | **Advantages** | **Disadvantages** |
|---|---|---|
| Slow Cooling | Stability from cracking relatively nontoxic cryoprotectants takes little technician time | Requires equipment, slow recovery, low applicability to tropical species. |
| Vitrification | No special equipment needed, fast procedure, fast recovery | Vitrification solutions are toxic to many plants, cracking is possible, requires careful timing of solution |
| Encapsulation-dehydration | No special equipment needed, nontoxic cryoprotectants, simple thawing procedures | Requires handling each bead several times, some plants do not tolerate the high sucrose concentrations. |
| Dormant bud preservation | Easy, useful for many temperate tree species | Requires freezing equipment, larger storage space, recovery requires grafting or budding, works best in cold temperate regions. |

Cryopreserved storage of important crop plants is being initiated in many countries throughout the world. The storage form varies with the crop type and the facilities available (Table 2). The numbers of accessions are low in most cases due to lack of funding for personnel to perform germplasm preservation at most sites.

Dormant branches of temperate trees are readily available from orchards and field collections. Storage of dormant wood requires more space than some other forms, however the ease of storage may make up for the space requirement. Grafting and budding skills are required for recovery of these materials. An 8-yr study of mulberry dormant buds found no change in the ability to form shoots with increased length of storage at –135 °C (23). Nearly 74% of 376 mulberry cultivars preserved for 5 yr had 50% or more shoot regrowth, and only 6% had regrowth of less than 30% of buds.

Meristems cultivated in vitro are widely tested in cryopreservation protocols because they are easy to multiply, available any time of year, easy to manipulate physically and physiologically, and can be recovered in culture. While in vitro systems require some additional input before storage, the ease of recovering and propagating the meristems is an advantage, and many accessions can be stored in a small dewar.

Embryonic axes are ideal storage forms for some species with recalcitrant seed. Removal and drying of the axes is time consuming, but not usually difficult, and the resulting propagules can be stored in a small space and recovered in vitro.

Somatic embryos are also a good form for cryopreserved storage. Many forest tree production systems depend on embryogenic callus to produce somatic embryos for testing Over 5000 genotypes of 14 conifer species are cryostored in one facility in British Columbia, Canada alone. Storage of these embryos allows for continued use of a line after an extended period of testing in the field (4). Long-term subculturing of embryogenic cultures can lead to somaclonal variation, so cryopreservation

of important lines from freshly initiated lines is very important.

Table 2. Cryopreserved collections of clonally propagated plant germplasm stored as dormant buds, in vitro meristems, or excised embryonic axes.

| Taxon | Country | Technique | Number Accessions/Replicates |
|---|---|---|---|
| **Dormant Buds** | | | |
| Apple | USA(NSSL) | E-D + CF | 2100 accessions |
| Elm | France (AFOCEL) | | 101 accessions |
| Mulberry | Japan (NIAR) - | CF | 45 accessions |
| **In Vitro Meristems** | | | |
| Apple | China | CF/E-D | 20 accessions/50 meristems each |
| Blackberry | USA(NCGR) | CF | 17 accessions/100 meristems each |
| Cassava | Columbia (CIAT) | E-D | 95 accessions/30 meristems each |
| Grass | USA (NCGR) | CF/E-D | 10 selections/100 meristems each |
| Hops | USA(NCGR) | CF | 2 accessions/100 meristems each |
| Pear | USA(NCGR) | CF | 106 accessions/100 meristems each |
| Potato | Germany (DSM/FAL) | | Droplet219 access ions/40-350 meristems |
| | Peru (CIP) | Vit | 197 accessions/250 meristems each |
| Ribes | USA(NCGR) | Vit | 5 accessions/100 meristems each |
| | Scotland (UAD) | E-D | 31 accessions/25-30 meristems each |
| **Embryonic Axes** | | | |
| Almond | India (NBPGR) | D-FF | 29 accessions/20 axes each |
| Citrus | India (NBPGR) | D-FF | 12 accessions of 6 species/50-100 |
| Hazelnut | USA(NCGR) | D-FF | 5 Species/100-300 axes each |
| Jackfruit | India (NBPGR) | D-FF/Vit | 3 accessions/25 axes each |
| Litchi | India (NBPGR) | D-FF | 2 accessions/30 axes each |
| Tea | India (NBPGR) | D-FF | 85 accessions/25 axes each |
| Trifoliate Orange | India (NBPGR) | D-FF | 1 accession/30 axes each |

Techniques: E-D - Encapsulation- Dehydration, CF - Controlled Freezing, Vit - Vitrification, Droplet - Droplet Freezing, D-FF - Dehydration-Fast Freezing.
Facilities: NSSL -National Seed Storage Laboratory-, AFOCEL-Association Foret-Cellulose-, NIAR -National Institute of Agrobiological Resources; NCGR - National Clonal Germplasm Repository-Corvallis-, CIAT- International Center for Tropical Agriculture-, DSM/FAL -Deutsche Sammulung von Mikroorganismen und ZellIkulturen/Institute fur Pflanzenbau, Bundesforxhungsanstalt fur Landwirtschaft -.UAD - University of Abertay Dundee-, CIP -International Center for the Potato, NBPGR - National Bureau of Plant Genetic Resources.

Pollen storage in liquid nitrogen is an important tool for plant breeders. Pollen storage requires drying pollen to low moisture contents. Pollen of many plants is easily stored and call be used for crosses that are not possible with fresh pollen due to differences in bloom time (15, 3 6).

## CONCLUSIONS

Cryopreserved collections of clonally propagated germplasm are now in place for many important horticultural crops. These initial collections provide the first long-term storage for clonally propagated crops. Cryopreservation should be considered a backup to field collections to insure against loss, Long-term viability studies monitoring the recovery of designated accessions will provide additional

information on the stability and viability of this storage form.

Curators planning cryostorage for their crops should first determine the most practical technique for their facility and the crops involved. Off site storage should be arranged well III advance with a reliable facility. A reciprocal exchange of LN-stored germplasm might be advantageous to two facilities needing off site locations. The management of records should be of prime importance since the recovery of plants from cryostorage will depend on knowing the proper techniques for thawing and regrowth of each accession.

Curators interested in using cryopreservation for long-term storage of germplasm collections should consider several steps before initiating storage. First, determine the best storage form for the crop in question; second, prioritize accessions to be stored third, determine the best technique to apply to these accessions- fourth, set up a database for needed information, fifth, make arrangements for offsite storage; sixth, plan long-term monitoring; and finally initiate storage.

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